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(54) Title: TELOMERASE ASSAY FOR PRECANCEROUS LESIONS OF THE UTERINE CERVIX

(57) Abstract

The present invention relates to a method of detecting or diagnosing a precancerous or cancerous lesion of the cervix, endocervix, vagina or vulva comprising measuring the telomerase activity of cells obtained from said tissue(s), wherein an increase in telomerase activity relative to the activity present in cells of non-cancerous tissue bears the presence of a cancerous or precancerous lesion.

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Description

TELOMERASE ASSAY FOR PRECANCEROUS LESIONS OF THE UTERINE CERVIX

1. INTRODUCTION

The present invention relates to a method of detecting or diagnosing a precancerous or cancerous lesion of the cervix, endocervix, vagina or vulva comprising measuring the telomerase activity of cells obtained from said tissue(s), wherein an increase in telomerase activity relative to the activity present in cells of non-cancerous tissue bears a positive correlation with the presence of a cancerous or precancerous lesion.

2. BACKGROUND OF THE INVENTION

Carcinoma of the uterine cervix is the third most frequent of the female genital cancers. Although there were only 13,000 cases of invasive cervical cancer predicted for 1996, this does not include more than 50,000 cases of carcinoma in situ and several times that number of cases of preinvasive dysplasias of the cervix. Of all the female genital cancers, only cervical cancer can be reliably prevented by use of an effective, inexpensive screening technique that allows detection of precancerous conditions that can be treated effectively so as to prevent the development of invasive cancer. Thus, the vast majority of deaths due to cervical cancer each year can be said to be preventable if women avail themselves of routine screening with cervical cytology.

Preinvasive cervical carcinoma is usually described by one of two different classifications, both of which are common usage. The first system divides lesions into dysplasias (mild, moderate and severe) and carcinoma in situ. The second system uses three divisions of the term cervical intraepithelial neoplasia (CIN-1, CIN-2 and CIN-3). This precursor stage of cervical carcinoma begins with minimal morphologic changes (CIN-1 or mild dysplasia) and progresses to the point that the entire epithelium from the

basement membrane to the surface is composed of malignant cells (CIN-3 or carcinoma in situ).

Preinvasive cervical carcinoma is detected by the Papanicolaou ("Pap") smear. The purpose of periodic cytologic screening by means of the Pap smear is to prevent invasive cervical cancer. Although microinvasive and early invasive cervical cancers will be detected and this early detection is valuable in decreasing the death rate from cervical cancer, the idea is to detect all cervical abnormalities in the premalignant stage and thus prevent invasive cervical cancer.

The Pap smear for precursors to cervical cancer has a number of serious flaws. These include: 1) a false negative rate which ranges from 6 to 55%, depending on the study cited (Singleton et al., 1995, CA-A Cancer Journal for Clinicians 45:305), 2) a high rate of equivocal diagnoses (i.e., Atypical Squamous Cells of Undetermined Significance ("ASCUS")) and (3) suboptimal sampling or staining technique. Only 10-45% of patients with ASCUS diagnosis were found to have squamous intra-epithelial lesion on follow-up, leading to unnecessary colposcopy and/or biopsies. Furthermore, a spectrum of medical conditions of the gynecologic tract which do not predispose to cancer can result in equivocal diagnoses such as ASCUS. These include infections from Candida or Trichomonas, nonspecific inflammatory responses, post-surgical and post-partum conditions, radiation or chemotherapy, and nonspecific changes in post-menopausal women.

Attempts have been made to automate or otherwise streamline the Pap smear method. These include: computerized image analysis of cells on Pap smears, both fully or semi-automated; detection of gene sequences from a group of human papilloma viruses (HPV) linked etiologically to squamous intraepithelial precancerous lesions (SIL), cervical, endocervical, vaginal and vulvar cancer. However, at present, the sensitivity of HPV genome detection is estimated to be only about 50 to 70%. In addition, HPV genome positivity is sometimes recorded in normal cervical or vaginal epithelium, constituting either false-positive testing, or alternatively interpreted as an indication of latent HPV infection of uncertain clinical significance.

Telomerase is an enzyme which functions to maintain the length of telomeres, specialized regions of chromosomes necessary for maintaining chromosomal stability. The cell can only afford to lose a finite number of these telomeres before sequences of the parent DNA are lost, resulting in chromosomal instability and subsequent cell death (Harley, 1991, Mutation Res. 256:271). In contrast, when cancer cells expressing telomerase divide, telomere length is maintained, rendering cancer cells resistant to cell senescence. Thus, telomerase activation may play a key role in transforming a mortal somatic cell into an immortal tumor cell (Haber, 1995, N. Engl. J. Med. 332:955).

3. SUMMARY OF THE INVENTION

The present invention relates to a method for detecting or diagnosing a precancerous or cancerous lesion of the cervix, endocervix, vagina or vulva, based on the presence of increased telomerase activity. It is based, at least in part, on the discovery that assaying cell samples from the surface of the cervix, endocervix, vagina and vulva for telomerase activity may be used as a sensitive alternative to the Pap smear for the detection of precancerous changes leading to cancer in these tissues. In particular, samples demonstrating a squamous intraepithelial precancerous lesion were found to express telomerase.

The present invention comprises the use of a molecular methodology for measuring telomerase activity (known as the "TRAP assay") for screening women for precancerous and cancerous lesions of the cervix, endocervix, vagina and vulva. The use of the TRAP assay avoids the slow, labor intensive, manual microscopic screening presently required by the Pap smear. The average Pap smear may contain epithelial cells, inflammatory cells, and microorganisms. It is thus not surprising that the average daily quota of Pap smears screened per day ranges from only 50 to a legal maximum of 100 smears per day per cytotechnologist. In contrast the TRAP assay for telomerase activity is objective.

4. DETAILED DESCRIPTION OF THE INVENTION

In view of the need for a sensitive and specific molecular assay for the detection of precancerous and cancerous changes of the female genital tissues, we have developed a method in which the level of telomerase activity is measured in cervical, endocervical, vaginal or vulvar cells harvested from a patient, for example, using a wooden spatula, cotton tipped swab, or brush-like device such as a cytobrush or cyto-broom, whereby an increase in telomerase activity bears a strong correlation to the presence of a precancerous or cancerous lesion of the cervix, endocervix, vagina or vulva.

Telomerase may be measured by any method known in the art, including the method set forth in the working example below. Assay of cervical, endocervical, vaginal and/or vulvar cells may be used, for example, and not by way of limitation, as a primary general population screen for precursors to cancer of these tissues; as a non-issue based, cytologic screen for such conditions; as a method for assessing the efficacy of treatment of squamous intraepithelial precancerous or cancerous lesions; as an alternative to Pap smear testing or as an ancillary test used in conjunction with Pap smears to assess patient subpopulations with high rates of atypias; and/or as an ancillary test for evaluating patients with an ASCUS diagnosis on Pap smear.

5. WORKING EXAMPLE

Patient Samples: Cells from cervix, endocervix (the inner lining of the cervix), and vagina were obtained by gentle scraping with a sterile cotton tipped swab. The swab was then immersed in sterile phosphate-buffered saline, set on ice for one hour, and then gently vortexed to release the cells. Suspended cells were then pelleted at 10,000 g for 30 min at 4°C, resuspended in ice-cold wash buffer [10 mM HEPES-KOH (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol], pelleted again, and then resuspended at 50 to 10^4 cells per 5 to 100 microliters of ice-cold lysis buffer [10 mM tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercapto-ethanol, 0.5% CHAPS (Pierce), 10% glycerol]. The resulting suspension was incubated for 30 min on ice, and then was centrifuged for 10 min in a microcentrifuge (14,000 g at

4°C), and the supernatant collected. The supernatant, referred to as the "sample extract", was then used in the TRAP assay.

Telomeric repeat amplification protocol (TRAP) assay: Assay tubes were prepared by lyophilizing 0.1 μg of CX primer (see below) onto the bottom of the tube and sealing it with Ampliwax (Perkin-Elmer). Fifty microliter TRAP reactions above the wax barrier contained 20 mM tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl. 0.005% Tween20, 1 mM EGTA, 50 µM dNTP, 0.1 µg of TS primer (see below), 2 U Ampl.Taq DNA polymerase (Perkin-Elmer), 1 µg of T4g32 protein (Boehringer Mannheim), bovine serum albumin (0.1 mg/ml), 1 to 15 µl of sample extract, and 0.2 µl of α^{32}P[dCTP] (Amersham) was added as described by Kim et al., 1994, Science 266:2011).

After 10 min at 23°C for extension of oligonucleotide TS by telomerase, tubes were transferred to a thermal cycler for 27 rounds at 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 1.5 minutes as previously described (Kim et al., 1994, Science 266:2011). The reaction was analyzed by electrophoresis in 0.5X tris-borate EDTA on 12% polyacrylamide nondenaturing gels and autoradiographed as previously described (Kim et al., 1994, Science 266:2011).

The TS primer is: 5'-AATCCGTCGAGCAGAGTT-3'

The CX primer is: 5'-$(CCCTTA)_3$CCCTAA-3'

These primers were previously described (Kim et al., 1994, Science 266:2011).

Results:

Telomerase assays were run on cell samples from 18 patients. Sixteen patients had cell samplings for telomerase immediately following routine Pap smears; two had cell sampling for telomerase after surgical removal of cervical tissue (from hysterectomy specimens). Of the eighteen samples assayed, thirteen showed positive telomerase activity, four showed negative telomerase activity, and one was equivocal. Of the thirteen positive telomerase samples, five had abnormal Pap smears (showing squamous intra-epithelial lesion or SIL); seven had abnormal but non-definitive Pap smears (diagnosis ranging from atypical squamous cells of undetermined significance or ASCUS to atypia and suspicious for SIL). A single telomerase positive sample (#P11) had a negative Pap

smear; however, this patient (#P11) had a history of SIL on biopsy followed by a recent abnormal Pap smear.

Of the four non-positive telomerase samples, all were negative on Pap smear and/or were negative with cervical tissue diagnosis. The one equivocal telomerase sample was from a patient with an abnormal but nondefinitive Pap smear (cannot rule out SIL or adenocarcinoma).

In this limited study, our false negative rate was 0% and our false positive rate was also 0%. Furthermore, in one sample (#P7), we were able to detect the presence of telomerase activity whereas the Pap smear was equivocal (ASCUS). However in this sample (P#7), the tissue revealed a squamous intraepithelial lesion/condyloma. Hence with this sample, the Pap smear was a false negative and the telomerase result was the true positive.

In conclusion, our findings reveal 1) telomerase assay is able to detect the earliest precancerous changes detectable by Pap smear (i.e. low grade SIL including mild dysplasia, condyloma, CIN 1; 2) telomerase assay thus far has no proven false negative results; 3) telomerase assay thus far has no proven false positive results; and 4) a positive telomerase assay may be indicative of the presence of a true precancerous lesion that is not defined by Pap smear.

Various publications are cited herein, including United States Provisional Patent Application No. 60/023,324, on which the present disclosure is based, which are hereby incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A method of identifying precancerous cells in a tissue selected from the group consisting of cervical, endocervical, vaginal and vulvar tissue, comprising measuring the level of telomerase activity in a cell sample of the tissue, wherein an increase in telomerase activity relative to the level of telomerase activity in non-cancerous cells of the tissue bears a positive correlation to the presence of a precancerous lesion.

2. A method of identifying cancerous cells in a tissue selected from the group consisting of cervical, endocervical, vaginal and vulvar tissue, comprising measuring the level of telomerase activity in a cell or cells of the tissue, wherein an increase in telomerase activity relative to the level of telomerase activating in non-cancerous cells of the tissue bears a positive correlation to the presence of a cancerous lesion.

3. The method of claim 1, wherein telomerase activity is measured by a telomeric repeat amplification protocol.

4. The method of claim 2, wherein telomerase activity is measured by a telomeric repeat amplification protocol.

5. A method of identifying a precancerous lesion in a subject at risk for a cancer selected from the group consisting of cervical cancer, endocervical cancer, vaginal cancer and bulbar cancer, comprising measuring the level of telomerase activity in a cell sample of a tissue selected from the group consisting of cervical, endocervical, vaginal and vulvar tissue, wherein an increase in telomerase activity relative to the level of telomerase activity in non-cancerous cells of the tissue bears a positive correlation to the presence of a precancerous lesion.

6. The method of claim 5, wherein teleomerase activity is measured by a telomeric repeat amplification protocol.

7. A method of identifying a cancerous lesion in a subject at risk for a cancer selected from the group consisting of cervical cancer, endocervical cancer, vaginal cancer and bulbar cancer, comprising measuring the level of telomerase activity in a cell sample of a tissue selected from the group consisting of cervical, endocervical, vaginal and

vulvar tissue, wherein an increase in telomerase activity relative to the level of telomerase activity in non-cancerous cells of the tissue bears a positive correlation to the presence of a cancerous lesion.

8. The method of claim 7, wherein telomerase activity is measured by a telomeric repeat amplification protocol.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/12193

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12Q 1/68; C12P 19/34
US CL :435/6, 91.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 91.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,489,508 A (WEST et al.) 06 February 1996, columns 8 and 12.	1-8
Y	KIM et al. Specific Association of Human Telomerase Activity with Immortal Cells and Cancer. Science. 23 December 1994, Vol. 266, pages 2011-2014, especially Table 1 and page 2014.	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"P"	document published prior to the international filing date but later than the priority date claimed		

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INTERNATIONAL SEARCH REPORT

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B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS; DIALOG: MEDLINE, CA, DERWENT PATENTS, BIOSIS, EMBASE, SCISEARCH
search terms: telomerase, cancer, neoplasm, precancer, tumor, cervix, cervical, vulvar, endocervical, bulbar, vaginal.